Functional Changes of Carboxymethyl Potato Starch by Conjugation with Whey Proteins

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Potato starch granules and whey protein were conjugated with a covalent bond to improve the function of starch. Slightly carboxymethylated starch (CMS) was prepared and dispersed in a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] solution. Whey protein isolate (WPI) was added, and the reaction mixture was incubated at 24 °C for 5 h, resulting in the formation of an acid–amide bond between CMS and WPI. WPI–CMS conjugate was recovered after thorough washing with a 0.9% NaCl solution and distilled water and then air-drying. The conjugation of WPI to the CMS granules was demonstrated by Coomassie Brilliant Blue staining, the WPI content of the conjugate being estimated to be about 6%. Conjugation markedly decreased the solubility and swelling of starch granules. DSC data revealed that the conjugate gelatinized at a higher temperature than CMS and ceased retrogradation. The digestibility of the conjugate with α-amylase and β-amylase decreased as compared with that of CMS and native starch. The retinol binding activity of the conjugate was confirmed by fluorescence titration.

Keywords: *Starch; neoglycoconjugate; functional improvement; acidic polysaccharide; protein conjugation; retinol binding; whey proteins; β-lactoglobulin*

INTRODUCTION

The high energy value and useful physical properties of starch have made it widely used in food and industrial applications as a thickener, water retention agent, colloidal stabilizer, gelling agent, bulking agent, adhesive, and so on. These functions of starch are brought about by its gelatinization and retrogradation behavior. This behavior is influenced by the kind of starch, the granule size, and the presence of a coexisting substance (Madeka and Kokini, 1992; Lisa and Jezof, 1992; Hamaker and Griffin, 1993). Hence, the function of starch is thought to be controllable by the choice of starch and granule size, and changeable by physical treatment (heating under dry or wet conditions) and chemical modification (starch ester, starch ether, cross-linking, etc.; Rutenberg and Solarek, 1984).

However, the recent increase in demand for functionality and application has made the characteristics of native starch and modified starch insufficient when considered from the viewpoint of material science. We considered that the conjugation of nonstarchy substances with starch would be important and effective for achieving significant functional changes of starch. Among the likely substances, protein is quite different from starch since it is a charged polymer.

We thus attempted to improve the functions of potato starch by conjugating starch and protein with a covalent bond. In this study, we chose whey proteins for conjugation, because whey proteins are rich in essential amino acids, have a high nutritional value, and possess useful properties for gelling, foaming, heat aggregation, emulsifying, and so on (Morr, 1982; Mulvihill and Kinsella, 1987; Vreeker et al., 1992). Whey proteins in bovine milk contain β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin, and immunoglobulin (Whitney et al., 1976). A half-weight of the whey proteins is β-LG, and about 25–30% weight is α-LA (Whitney et al., 1976). It is also well-known that β-LG has retinol binding ability (Futterman and Heller, 1972) and that α-LA participates in the lactose synthetase reaction (Fitzgerald et al., 1970). We thought that it would be possible to provide starch with these functions by conjugation with whey proteins and this choice would be appropriate as a model for changing the functions of starch.

Our objectives were to raise the thermal stability of starch, to depress its retrogradation, to reduce its digestion with amylase, and to provide starch with some biological functions. In this work, the carboxymethyl groups required for conjugating with WPI were slightly introduced to potato starch as the first step. The resulting slightly modified carboxymethyl starch (CMS) was conjugated with WPI by water-soluble carbodiimide, and the functional changes in the conjugated potato starch were investigated in detail.

MATERIALS AND METHODS

Materials. Potato starch was repeatedly washed with redistilled water at 24 °C and then air-dried (14.7% final water content). WPI was supplied by Meiji Milk Products Co. (Tokyo, Japan), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride was purchased from Dojindo (Kumamoto, Japan). The other reagents were of special grade commercially available.

Carboxymethylation of the Starch. Potato starch (2 g) was dispersed in 32 mL of methyl alcohol containing 0.6 g of monochloroacetic acid and 0.7 g of sodium hydroxide and shaken gently at 40 °C for 5–48 h. The reaction mixture was neutralized to pH 6.5 with acetic acid after cooling to room temperature and then filtered. After a wash with methyl alcohol, CMS was obtained by air-drying, the degree of modification being determined by hydrochloride–methyl alcohol titration (Smith, 1967).

Preparation of the WPI–CMS Conjugate. The WPI–CMS conjugate was prepared by referring to the method of Hoare and Koshland (1967). CMS (0.2 g) was dispersed in 4

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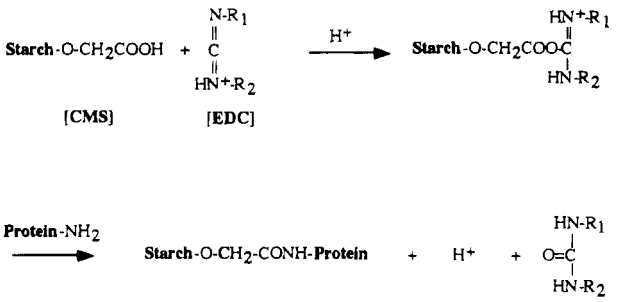
mL of a 5% EDC solution while the reaction mixture was maintained at pH 7.0, before 8 mL of WPI solution (25 mg/mL) was gradually added. The reaction mixture was incubated at 24 °C for 5 h with gentle stirring. After the reaction was stopped by adding 0.72 mL of 1 M acetic acid, the reaction mixture was washed five times with a 0.9% NaCl solution and distilled water and centrifuged at 5000 rpm for 10 min. The conjugate was obtained by air-drying.

The expected mechanism of conjugation between CMS and WPI by using EDC is as follows:



Treatment with Protease. The conjugate was digested with Actinase (Kaken Pharmacy Co., Tokyo, Japan) as a protease as follows. The conjugate (0.1 g) was dispersed in 100 mL of a 0.1 M Tris-HCl buffer at pH 7.8 containing 5 mM calcium chloride and digested twice with 5 mg of Actinase at 30 °C for 24 h. After five washings with fresh buffer and centrifugation at 5000 rpm for 5 min, the sample was incubated in 50 mL of 0.1 M EDTA at pH 7.0 at room temperature for 2 h to eliminate calcium, washed repeatedly with distilled water, and finally recovered by air-drying.

Microscopic Observation. CMS or the conjugate (10 mg) was heated in 1 mL of distilled water at 70 °C for 10 min and stained with 1 mL of a Coomassie Brilliant Blue solution (CBB, 10 mg/mL) after being cooled to room temperature. After 15 min, the sample was washed with distilled water by centrifuging three times at 3000 rpm for 5 min. The sample was observed under a polarizing microscope (Model PM-10AD sp, Olympus, Tokyo, Japan).

Solubility. CMS or the conjugate (10 mg) was suspended in 5 mL of distilled water and heated at constant temperature (50, 60, 80, or 95 °C) for 1 h with stirring. The solubility was evaluated by determining the saccharide concentration of the centrifuged supernatant (at 18 000 rpm for 10 min) by the phenol–sulfuric acid method (Dubois et al., 1956).

Differential Scanning Calorimetry (DSC). CMS or the conjugate was subjected to DSC (Seiko SSC-5020 DSC 100, Tokyo, Japan) as described previously (Takahashi et al., 1988). From the DSC curve, the gelatinization temperatures [onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c)] and gelatinization enthalpy were evaluated as characteristics of the gelatinization process.

The conjugate, CMS, and native starch gelatinized with the DSC apparatus were preserved at 4 °C for 7 days prior to a second analysis by DSC. The ratio of the second gelatinization enthalpy to the first could be regarded as the retrogradation degree (Nakazawa et al., 1985).

Digestion with α-Amylase. The digestibility of the samples with α-amylase (EC 3.2.1.1, Sigma, St. Louis, MO) was measured. A sample (2 mg) was dispersed in 1.8 mL of a 0.02 M sodium citrate buffer at pH 6.5 containing 0.1 M sodium chloride (the citrate buffer) and was heated at 100 °C for 10 min. After the mixture was cooled to room temperature, 0.002 unit of α-amylase in 0.2 mL of the citrate buffer was added and the reaction mixture was incubated at 30 °C for 0–120 min.

Digestibility was evaluated by determining the saccharide concentration of the filtrate after each period of digestion by the phenol–sulfuric acid method (Dubois et al., 1956).

Digestion with β-Amylase. The digestibility of the samples with β-amylase (EC 3.2.1.2, Sigma) was also measured. A sample (2 mg) was dispersed in 1.9 mL of distilled water; 0.1 mL of a 1 M acetate buffer at pH 6.0 (the acetate buffer) was added, and the sample was heated at 100 °C for 10 min. After the mixture was cooled to room temperature, 0.1 unit of β-amylase in 0.1 mL of the acetate buffer was added, and the reaction mixture was incubated at 30 °C for 0–120 min.

Digestibility was evaluated by determining the saccharide concentration of the filtrate after each period of digestion by the phenol–sulfuric acid method (Dubois et al., 1956).

Competitive Enzyme-Linked Immunosorbent Assay (ELISA). To confirm the presence of native β-lactoglobulin (β-LG) in the conjugate, competitive ELISA was carried out by using monoclonal antibody (mAb) 61B4 as described previously (Kaminogawa et al., 1989; Hattori et al., 1993). MAb 61B4 reacts preferentially to native β-LG but much more weakly to the denatured form of β-LG. The epitope for mAb 61B4 has been functionally determined to be 125Thr–135Lys (Kaminogawa et al., 1989). A 0.01% β-LG solution (100 μL, dissolved in PBS) was added to the wells of a 96-well microtitration plate (Nunc, Roskilde, Denmark), and the plate was incubated at 25 °C for 2 h. After the antigen solution had been removed and washed three times with 125 μL of PBS-Tween (0.05% Tween 20 in PBS), 125 μL of a 1% ovalbumin solution was added. The plate was again incubated at 25 °C for 2 h and washed. The β-LG solution (50 μL) or the same volume of the conjugate suspension at suitable concentrations was added to the wells just before 50 μL of mAb 61B4 solution was added, and the plate was further incubated at 25 °C for 2 h. After removing the solution and washing, 100 μL of alkaline phosphatase-labeled goat anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) diluted with PBS-Tween was added to the plate, which was again incubated at 25 °C for 2 h. After the wells were washed, 100 μL of 0.1% *p*-nitrophenyl phosphate disodium salt in a 1 M diethanolamine hydrochloride buffer at pH 9.8 was added as a substrate and incubated at 25 °C for 30 min. The reaction was stopped by adding 20 μL of 5 M sodium hydroxide to the wells, and the absorbance was determined at 405 nm. The β-LG content in the conjugate was calculated from the binding curve of mAb 61B4 to β-LG and from the concentration of the conjugate in the suspension used for ELISA (500 μg/well).

Measurement of the Retinol Binding Activity of the Conjugate. The retinol binding activity of the conjugate was measured by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993). At first, the fluorescence titration curve for β-LG with retinol was obtained as follows. Two milliliters of PBS solution containing 2.0 mg (109 nmol) of β-LG was added to a cuvette. Small increments (5 μL at a time) of 2.26×10^{-4} M retinol (Sigma) in ethanol were added to the cuvette with a micropipet. The mixture was thoroughly mixed and allowed to equilibrate for 1 min before the fluorescence intensity was recorded. The fluorescence was measured by a Shimadzu RF-510 fluorometer (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm.

CMS or the conjugate (30 mg) was dispersed in 1.7 mL of PBS; 300 μL of 2.26×10^{-4} M retinol in ethanol was gradually added with a micropipet, and the suspension was centrifuged at 3000 rpm for 5 min. The supernatant (1 mL) was added to 1 mL of a solution in a cuvette containing 2 mg (109 nmol) of β-LG in PBS with gentle stirring, and the fluorescence was measured. The difference in fluorescence between CMS and the conjugate was calculated as the retinol binding ability of the conjugate.

At the end of the various titrations, the ethanol concentration amounted to less than 7% and is assumed to have had no effect on the measurement system. The solution remained clear without any turbidity throughout the entire range of investigated concentrations.

RESULTS AND DISCUSSION

Features of CMS. The degrees of modification (DM) of the CMS preparations were evaluated to be 13, 25, 34, 49, and 59 (carboxymethyl residues/1000 glucose residues) depending on the reaction time. The gelatinization temperatures and enthalpy values of CMS

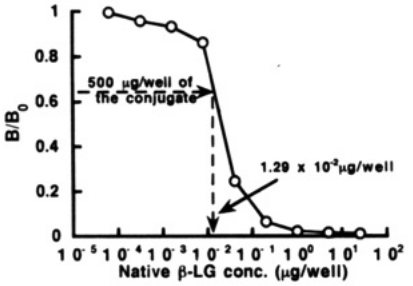


Figure 1. Detection of native β-LG in the WPI–CMS conjugate with mAb 61B4 by competitive ELISA. Competitive ELISA was performed as described previously (Kaminogawa et al., 1989; Hattori et al., 1993). The β-LG content in the WPI–CMS conjugate was calculated from the binding curve of mAb 61B4 to β-LG and the concentration of WPI–CMS in the suspension used for ELISA (500 μg/well).

decreased from 51.6 °C and 15.4 mJ/mg to 37.4 °C and 4.1 mJ/mg with increasing DM from 13 to 59 (data not shown). Since the hydroxy groups of CMS were partially substituted by carboxymethyl groups, it is considered that the electrostatic repulsion by the carboxyl groups resulted in a decrease in the intermolecular cohesion of CMS. The solubility values of these CMS preparations in distilled water at 60 °C for 1 h (without stirring) were about 17%, 20%, 25%, 30%, and 33%, respectively, which are very much higher than the value (4%) for native starch. The CMS preparations with DM above 49 were partially soluble in distilled water at room temperature, while CMS with DM 34 was essentially the same as native potato starch in its granular shape and polarization pattern (data not shown). To prepare the insoluble conjugate, we chose CMS with DM 34, which was insoluble in cold water with the highest carboxyl group content.

Conjugation of CMS and WPI. The conjugated amount of WPI was estimated to be 5.8% by determining the protein concentration of the centrifuged supernatant of the reaction mixture by the absorbance at 280 nm. The result of competitive ELISA indicates that 1 g of the conjugate contained 26 μg of native β-LG (Figure 1), this content being very low. Since the conjugation of β-LG with carboxymethyl dextran showed little conformational change in β-LG (Hattori et al., 1994), it is considered that Lys residue(s) around the epitope for mAb 61B4 was (were) used for conjugation with CMS or that steric hindrance to the binding of mAb 61B4 resulted from the conjugation. The conjugate could be stained with CBB and swollen in a limited way by heating, while CMS and the conjugate after removal of the conjugated WPI with Actinase could not be stained and extremely swelled (Figure 2). This demonstrates that WPI could be conjugated with the CMS granules, especially to the surface area of CMS, with water-soluble carbodiimide, and it is to be expected that many of the properties of CMS would have been changed.

Changes in Solubility, Gelatinization, and Retrogradation Properties. Figure 3 shows the effect of conjugation on the solubility of CMS. The solubility of the conjugate at 95 °C was only 6%, while the values for CMS and native starch were about 53% and 47%, respectively, showing a sharp contrast. It is considered that bonding of WPI to the surface of the CMS granules resulted in this marked decrease in solubility.

The DSC curves indicated that the conjugate gelatinized at 50.7 °C (peak temperature, T_p), which is about 8 °C higher than T_p for CMS (Figure 4; Table 1). The gelatinization temperature of CMS in WPI solution was

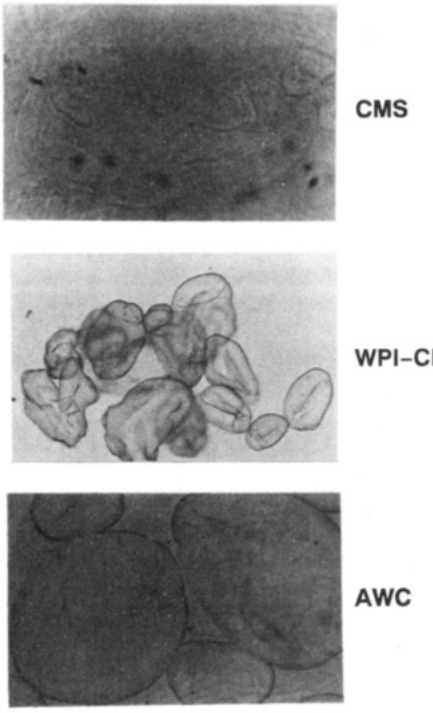


Figure 2. Polarizing micrographs of the WPI–CMS conjugate, CMS, and WPI–CMS digested with Actinase (AWC) after heating at 70 °C for 10 min and staining with CBB. Direct magnification: 100×. (This figure is reproduced here at 50% of its original size.)

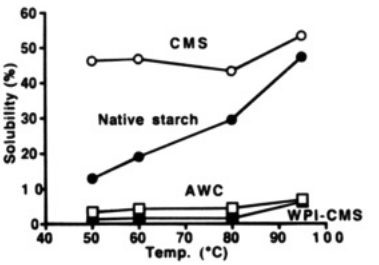


Figure 3. Solubility of the WP–CMS conjugate. Samples were suspended in 5 mL of distilled water and heated at constant temperature (50, 60, 80, or 95 °C) for 1 h with stirring. The solubility was evaluated by determining the saccharide concentration for the centrifuged supernatant (at 18 000 rpm for 10 min) by the phenol–sulfuric acid method (Dubois et al., 1956). AWC: WPI–CMS digested with Actinase.

somewhat higher than that of CMS in distilled water. Since CMS in WPI solution contained a high concentration (6%) of protein and the viscosity of the solution was high, CMS would have been hard to gelatinize. When the conjugate was digested with Actinase, T_p was 46.0 °C, which is close to that of CMS. These results indicate that conjugation of WPI to CMS resulted in an increase in the gelatinization temperature. The effect of conjugating WPI to CMS is similar to the fact that the gelatinization temperature of starches in foods is higher than that of the isolated material (Wada et al., 1979). The gelatinization enthalpy of CMS rather increased from 14.2 to 17.0 mJ/mg by conjugation, being close to 19.5 mJ/mg for native starch.

The retrogradation degree for CMS, which was evaluated by the ratio of the second gelatinization enthalpy to the first, was very small when compared with that of native starch (Figure 4; Table 1). As compared with the retrogradation degrees of 39% for native starch and of 14% for CMS, the conjugate showed a low retrogradation value of 12%, this value being much less than the value for native starch and slightly less than that for

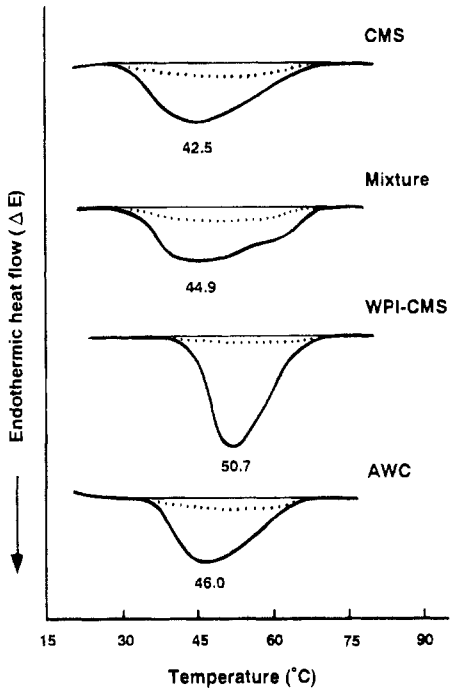


Figure 4. DSC curves for the WPI–CMS conjugate, CMS, a mixture of WPI and CMS, and WPI–CMS digested with Actinase (AWC). Conditions; DSC, Seiko 5020 DSC 100; heating rate, 2 °C/min; atmosphere, He, 40 mL/min; cell, anodized aluminum cell. —, first gelatinization; - - -, second gelatinization (after preserving at 4 °C for 7 days).

Table 1. Gelatinization Temperature, Enthalpy, and Retrogradation Degree of the WPI–CMS Conjugate

	gelatinization temp (°C)			ΔH	retrogradation
	T_o	T_p	T_c	(mJ/mg)	degree (%)
native starch	58.9	61.8	66.9	19.5	39.0
CMS	33.5	42.5	65.1	14.2	14.1
WPI–CMS	45.0	50.7	63.6	17.0	11.8
WPI + CMS mixture[a]	40.8	44.9	67.3	13.3	13.3
AWC[b]	38.0	46.0	62.2	13.1	13.1

[a] WPI + CMS mixture: a mixture of WPI and CMS, the ratio of which is the same as that of the WPI–CMS conjugate. [b] AWC: WPI–CMS digested with Actinase.

CMS. Thus, the conjugate was hard to retrograde, indicating that the bonding of WPI to CMS might have restricted the mobility of starch chains and resulted in inhibition of their rearrangement.

Digestibility with α-Amylase and β-Amylase. The digestibility of the conjugate with α-amylase and β-amylase compared with that of native starch and CMS is shown in Figure 5. CMS was more digestible than native starch with α-amylase since CMS had higher solubility and swelling power than native starch. However, the digestibility of CMS with β-amylase was similar to that of native starch. Since β-amylase is an exoamylase, the carboxymethyl groups in CMS might have inhibited the digestion with β-amylase by steric hindrance. The WPI–CMS conjugate was the most indigestible with both α-amylase and β-amylase when compared with native starch and CMS. This lower digestibility of the conjugate with α-amylase and β-amylase might have been caused by the lower solubility and swelling power of the conjugate. These results indicate that the conjugation of WPI to CMS could endow CMS with the characteristics of an indigestible polysaccharide.

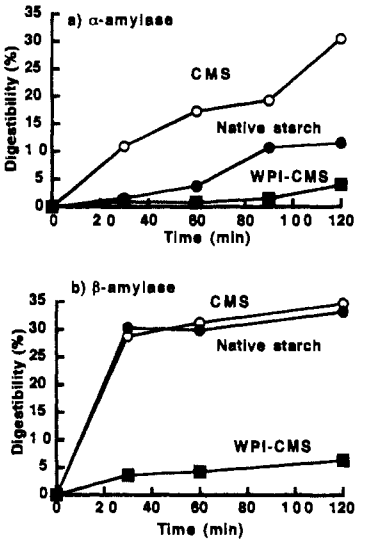


Figure 5. Digestibility of the WPI–CMS conjugate by α-amylase and β-amylase. Samples were heated at 100 °C for 10 min. After the samples had cooled to room temperature, α-amylase or β-amylase was added and the reaction mixture was incubated at 30 °C for 0–120 min. Digestibility was evaluated by determining the saccharide concentration of the filtrate after each period of digestion by the phenol–sulfuric acid method (Dubois et al., 1956).

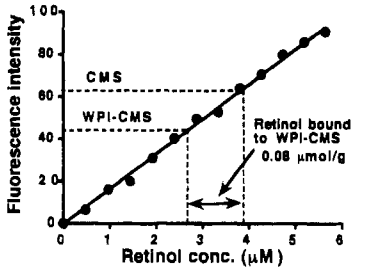


Figure 6. Retinol binding ability of the WPI–CMS conjugate.

Retinol Binding Activity of the Conjugate. It is well-known that β-LG, the major protein of WPI, has retinol binding ability. The possibility of whether this retinol binding activity of β-LG would be endowed in the conjugate was investigated by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993). The difference in fluorescence between CMS and the WPI–CMS conjugate was calculated as the retinol binding ability of the conjugate. It was clarified that 1 g of the conjugate bound 0.08 μmol of retinol (Figure 6). Since the native β-LG monomer binds one molecule of retinol (Fugate and Song, 1980) and the denatured form of β-LG cannot bind retinol (Hattori et al., 1993), it was also clarified that β-LG was conjugated to CMS in the native form. By conjugating with proteins, starches could be endowed with the biological activity of proteins.

Concluding Remarks. In this study, we prepared WPI–CMS conjugate with an acid–amide bond by using water-soluble carbodiimide. The functions of potato starch could be changed by conjugating with WPI. The solubility, swelling power, and digestibility with α-amylase and β-amylase were much less when compared with the values for CMS, while the thermal stability of CMS was enhanced by the bonding of WPI. As a new physiological function, retinol binding ability was endowed to CMS by the conjugation of WPI. It is hoped that the WPI conjugate prepared in this study will contribute to the development of novel applications for starch.

ABBREVIATIONS USED

CMS, carboxymethylated starch; WPI, whey protein isolate; CBB, Coomassie Brilliant Blue; DSC, differential scanning calorimetry; β-LG, β-lactoglobulin; α-LA, α-lactalbumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween 20; DM, degrees of modification.

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